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A retrovirus carrying the promyelocyte–retinoic acid receptor PML-RARα fusion gene transforms haematopoietic progenitors *in vitro* and induces acute leukaemias

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The promyelocyte (PML)–retinoic acid receptor α (RARα) fusion gene results from a t(15;17) chromosome translocation in acute promyelocytic leukaemia. We have analysed the oncogenic potential of the human fusion PML–RARα product in chicken using retrovirus vectors. We show that PML–RARα transforms very early haematopoietic progenitor cells *in vitro* and induces acute leukaemias. Neither PML nor RARα domains alone achieve such a transformation. The PML–RARα viruses recovered from the transformed cells carry two point mutations in the PML domain, one of which alters both the pattern of intracellular localization of the fusion protein and its functional interference with AP-1, thus defining an essential domain in PML for oncogenic transformation.

Keywords: haematopoietic differentiation/leukaemic transformation/nuclear hormone receptor/PML–RARα

Introduction

Chromosome translocations result in many cases in the fusion of two genes into a unique transcription structure which then encodes a fusion product. In man, many such rearrangements have been found to be associated with leukaemias and have led to the identification of new genes at the translocation breakpoints (for a review see Sawyers *et al.*, 1991). The genes that take part in chromosome rearrangements in human acute leukaemias are supposed to be master genes involved in the implementation of the differentiation program and many of them encode transcription factors (for a review see Rabbits, 1991). However, for many of these genes their direct role in the induction of leukaemic transformation has not yet been demonstrated.

Acute promyelocytic leukaemia (APL) is characterized by a specific t(15;17)(q22;q21) chromosome translocation which fuses the promyelocyte (PML) gene carried on chromosome 15 to the gene encoding the retinoic acid receptor α (RARα) on chromosome 17 (Borrow *et al.*, 1990; de Thé *et al.*, 1990; Kakizuka *et al.*, 1991; Kastner *et al.*, 1992; Pandolfi *et al.*, 1992). The rearranged locus encodes a fusion PML–RARα product which is found in all APL patients. A RARα–PML product resulting from the reciprocal t(15;17) translocation is detected in many, but not all patients, suggesting that it does not play a crucial role in APL (Alcalay *et al.*, 1992; Chang *et al.*, 1992).

The PML product belongs to a novel family of nuclear proteins which share common structural features including, at the N-terminus, a cysteine/histidine-rich cluster (the so-called RING domain), followed by one or two additional cysteine/histidine-rich regions referred to as B box motifs. The C-terminal moiety encloses a predicted coiled-coil domain with potential for dimerization (for a review see Reddy *et al.*, 1992). The PML protein is concentrated within well-depicted subnuclear structures, called nuclear bodies, in association with several other proteins identified as auto-antigens in some human autoimmune diseases (Dyck *et al.*, 1994; Koken *et al.*, 1994; Weis *et al.*, 1994).

In the PML–RARα fusion protein, the cysteine/histidine-rich domains of PML are preserved and fused upstream of the DNA binding domain of RARα, which remains unaltered in its dimerization and ligand binding domains. The transcriptional activity of the RARα domain is altered by its fusion with PML. Depending on the cells and the reporter promoters used for the assay, PML–RARα showed a reduced or enhanced transcriptional activity compared with RARα (de Thé *et al.*, 1991; Kakizuka *et al.*, 1991; Pandolfi *et al.*, 1991; Kastner *et al.*, 1992). In APL cells, the PML–RARα protein disrupts the nuclear bodies, which reconstitute upon treatment of the cells with all-*trans* retinoic acid (RA) (Dyck *et al.*, 1994; Koken *et al.*, 1994; Weis *et al.*, 1994). This treatment induces the differentiation of the leukaemic cells into mature granulocytes and is used as a therapeutic for APL patients (Huang *et al.*, 1988; Warrell *et al.*, 1991).

So far, there is no direct evidence that PML–RARα is directly and solely responsible for the leukaemic transformation of myelocytic progenitors. The fusion protein has been shown to block the differentiation of myelocytic or erythrocytic leukaemic cell lines when it is artificially expressed in these cells (Grignani *et al.*, 1993, 1995; Rousselot *et al.*, 1994). It seems that PML–RARα may contribute to both leukaemic cell differentiation arrest and response to RA (Grignani *et al.*, 1993).

Here we have analysed the oncogenic potential of the PML–RARα product in haematopoietic progenitors in the chicken using an efficient retrovirus vector strategy. We have demonstrated that the fusion protein transforms very early haematopoietic progenitor cells *in vitro* and that it induces an acute leukaemia in chickens. In the transformed cells we found that the fusion protein contains two point mutations in the PML domain which seem to be required

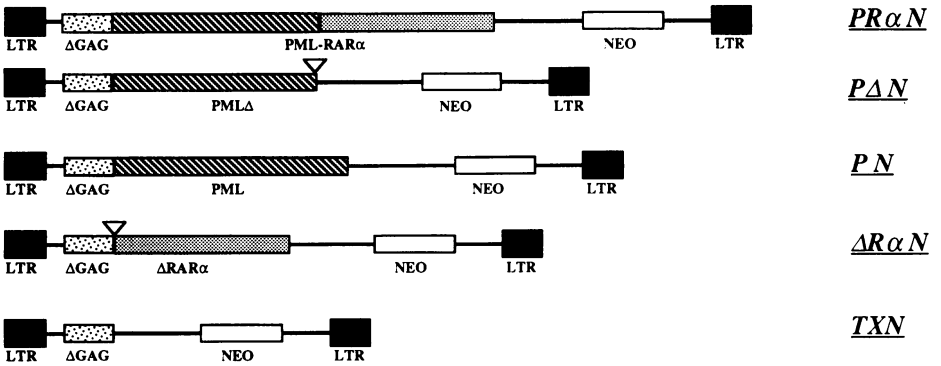


Fig. 1. Genomic structure of the different viruses. The open triangles indicate deletion compared with the sequence of the normal product.

for leukaemic transformation. Therefore these data provide an experimental biological assay to dissect the role of PML–RARα in leukaemic transformation.

Results

Structure of retrovirus vectors

Preliminary attempts to construct replication-competent retrovirus vectors expressing PML, RARα or PML–RARα native products were unsuccessful. All the vectors underwent rearrangements leading to the absence of expression of the carried foreign sequences. Then we decided to construct defective retroviruses in which the PML, RARα or PML–RARα coding sequences were fused with the gag sequence, so as to produce gag fusion products. The vectors were constructed from an avian erythroblastosis virus (AEV) backbone genome containing a Neo^R gene, in which the preserved gag sequences covering the p19 and the first 38 amino acids of p10 were identical to those found in many natural gag–v-onc fusion products (Bishop and Varmus, 1985). The following human cDNA sequences were fused in-frame with this gag residual sequence: the entire PML cDNA in virus PN, the 3′-truncated PML coding sequence derived from the long form of PML–RARα (de Thé *et al.*, 1990) in virus PΔN, the 5′-deleted RARα coding sequence derived from the same PML–RARα in virus ΔRαN, and the full long form of the PML–RARα coding sequence in virus PRαN. All these genes were expressed from the AEV long-terminal repeat in the form of genomic RNAs. The genomic structures of the viruses are shown in Figure 1. We checked that all the retrovirus vectors correctly transferred and expressed the carried ectopic sequences by analysing the size of the proteins synthesized in infected chicken embryo fibroblasts (CEFs; data not shown).

Although fusion with gag does not alter the intracellular localization of many natural nuclear viral oncoproteins, we analysed whether the gag–PML–RARα product would exhibit the specific pattern of nuclear localization of the native PML–RARα protein. Cos-7 cells were transfected with expression vectors of either PML–RARα or gag–PML–RARα (data not shown). Both the PML–RARα and the gag–PML–RARα proteins were concentrated mainly in the nucleus within numerous microstructures, as described previously for PML–RARα in transfected cells (Kastner *et al.*, 1992; Dyck *et al.*, 1994; Koken *et al.*, 1994; Weis *et al.*, 1994). Moreover, as reported earlier for PML–RARα, the multigranular staining of gag–PML–RARα was replaced by a more homogeneous nuclear labelling after treatment of the transfected cells with 10^{-6} M all-*trans* RA (data not shown).

Next we verified whether the fusion with gag might alter the transcriptional activity of PML–RARα. As reported previously for PML–RARα (Kakizuka *et al.*, 1991), the gag–PML–RARα product could transactivate a reporter plasmid containing a chloramphenicol acetyltransferase (CAT) gene driven by an SV40 promoter fused with a single βRARE (pΔSV–βRE) in HeLa cells (see Figure 4B). We also investigated the functional interference of gag–PML–RARα with the AP-1 transcription factor. Doucas *et al.* (1993) have shown that cells overexpressing PML–RARα exhibit a low AP-1 activity in the absence of RA, and that this activity is partially restored in the presence of RA. We observed a similar effect of gag–PML–RARα under these same conditions (see Figure 4B).

Altogether, these data show that the virus vectors express the expected proteins and that the fusion with gag does not alter the intracellular localization, the transcriptional activity of PML–RARα on a natural RARE and its functional interference with AP-1.

Transformation of chicken bone marrow cells (BMC) in vitro by the virus PRαN

To investigate the oncogenic properties of the retrovirus vectors on haematopoietic cells, BMCs from 17–19 day old chicken embryos were infected *in vitro* with similar doses of the respective virus and then seeded in methyl cellulose cultures containing G418. In the cultures infected with either TXN, PN, PΔN or ΔRARα only a few red bursts developed from the infected normal erythrocytic progenitors BFU-E which were not affected in their differentiation. In contrast, in the PRαN-infected cultures, large white and spherical colonies appeared after 7 days at a frequency of ~200 colonies per 10^6 seeded cells. These colonies were similar in shape and size to the transformed colonies observed after the infection of chicken BMCs with the leukaemic retroviruses AEV or E26 (Samarut and Gazzolo, 1982; Moscovici *et al.*, 1983). After seeding the colonies in liquid culture, their cells grew with an 18–24 h doubling time (data not shown). In contrast to haematopoietic cells transformed by the v-erbA oncogene (Pain *et al.*, 1991), these cells did not require transforming growth factor α for their growth (data not shown). They could be passed and grown for nearly 30 generations, after which they became senescent. These cells could also grow in low-serum medium (data not

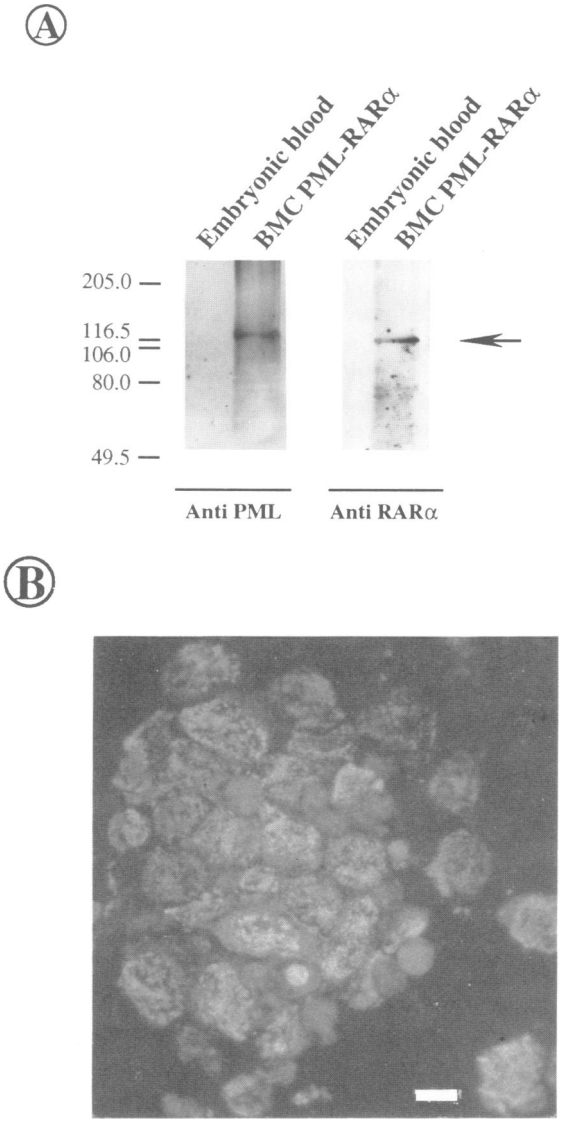


Fig. 2. Expression of gag–PML–RARα in BMCs. (**A**) The detection of gag–PML–RARα product in BMCs infected with PRαN. The same blot was revealed with either an anti-PML or an anti-RARα antibody. 10 μg total protein of cell extract were loaded. (**B**) Analysis of the intracellular localization of gag–PML–RARα product in BMCs infected with PRαN. The fusion protein was detected with an anti-PML antibody. Bar scale, 5 μm.

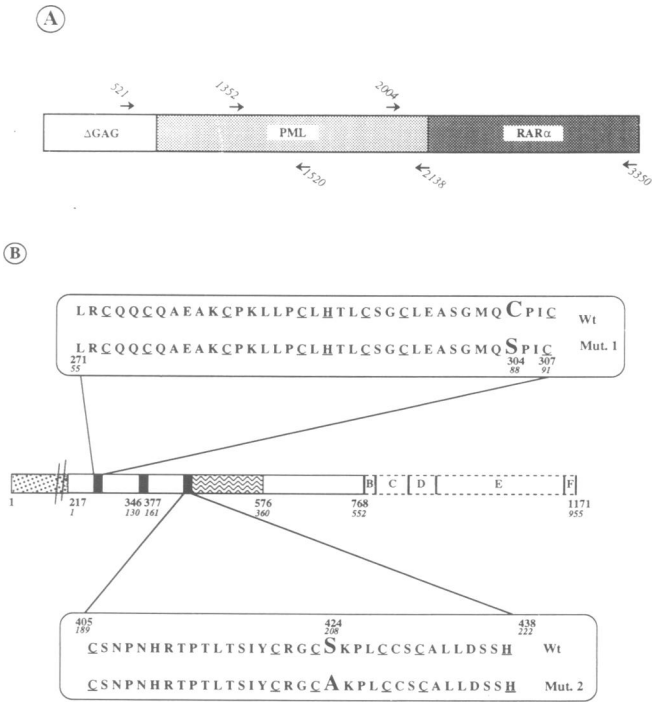


Fig. 3. Identification of point mutations in gag–PML–RARα in transformed cells. (**A**) Schematic diagram of gag–PML–RARα showing the PCR primers. The following pairs of primer were used for amplification on the integrated proviruses: *521* with *1520*; *1352* with *2138*; and *2004* with *3350*. (**B**) The sequence of the PML domain in integrated PRαN provirus in transformed haematopoietic cells. The gag sequence of the virus is represented by the stippled box. In the PML domain the three cysteine/histidine-rich motifs (black boxes) and the coiled-coil region (wavy box) are depicted. The cysteine/histidine residues conserved in the Zinc finger-like region are underlined. The truncated RARα moiety is shown by dashed lines. The point mutations are indicated in larger sized characters. Numbers of the amino acid residues of gag–PML–RARα protein (plain characters) and the numbers corresponding to the native PML–RARα protein (italics) are indicated.

shown). These data suggest that the PRαN-transformed cells exhibit a strong proliferative ability and low growth factor requirement.

The expression of the gag–PML–RARα product in these transformed cells was analysed by a Western blot, which revealed the presence of a protein of the expected size (Figure 2A). The protein was revealed by immunofluorescence in almost all of the cells, and was found predominantly in the nucleus with a microparticulate distribution, a pattern reminiscent of PML–RARα in APL cells (Figure 2B).

The gag–PML–RARα product in transformed haematopoietic cells contains two point mutations

As minute rearrangements in the PML–RARα sequence might account for the oncogenic activity of the virus, we sequenced this gene within the provirus in haematopoietic cells transformed *in vitro* by PRαN in two independent experiments. Genomic DNA was isolated from the transformed cells and several fragments of the gag–PML–RARα gene were amplified by PCR using the oligonucleotide pairs indicated in Figure 3A. The amplified fragments were then sequenced, and the full sequence of the PML–RARα coding domain was determined. Whereas both transformed cell populations were polyclonal, their integrated viral genome unambiguously showed a uniform sequencing pattern with two mutations at residues 304 and 424 (Figure 3B). The first point mutation changes cysteine 304 in the PML RING domain region (residue 88 of the PML–RARα product) into a serine. Another mutation changed the serine at position 424 (position 208 of the PML–RARα product) into an alanine. This second mutation is localized in the third cysteine/histidine-rich region of PML, named the B2 box (Reddy *et al.*, 1992).

We tried to reconstitute the genesis of the two mutations. First, we checked that none of the mutations were present in the transfected plasmids. To verify the presence of the mutations in viruses collected from the initial batch of transfected CEFs, the collected virus suspension was used to infect fresh CEFs in which we sequenced the integrated proviruses. The population of PCR-amplified fragments unambiguously showed a homogeneous mutant pattern

encoding a serine at position 304, but no mutation was found at residue 424. In another independent transfection which failed to produce any detectable vector particles, we did not find a mutation in the transfected gag–PML–RARα sequence. These observations suggest that the mutation of cysteine 304 into a serine may favour the production of infectious particles carrying the retrovector genome.

Two independent transformation assays of BMCs were performed with the supernatant containing the vector with the mutation at residue 304. The gag–PML–RARα sequence integrated in the transformed cells was then determined for each experiment. Whereas both transformed cell populations were polyclonal, their integrated viral genomes unambiguously showed a uniform sequencing pattern, with the two mutations at residues 304 and 424. Two hypotheses can be proposed to explain the generation of mutation 424 in the transformed haematopoietic cells: either rare integrated proviruses in the virus-producing CEFs carried the two mutations and then released a few double-mutant virus particles which were then selected in the bone marrow transformation assay, or mutation 424 appeared during infection of the haematopoietic progenitors. In any case, these data suggest that the mutation of the serine into an alanine at position 424 could be necessary for the transformation of haematopoietic cells.

Effects of the mutations on the intracellular localization and transcriptional properties of the fusion protein

To determine the effects of the two mutations on the biochemical properties of the gag–PML–RARα product, we constructed expression plasmids of single- or double-mutant proteins. The plasmids gag–PML–RARαWM, gag–PML–RARαMW and gag–PML–RARαMM carry, respectively, the single mutation 424, the single mutation 304 and the double 304–424 mutation.

First we investigated the intracellular localization of the mutated forms in Cos-7 cells by an immunofluorescence study performed 2 days after transfection of the respective expression vectors. As shown in Figure 4A, the gag–PML–RARαWM protein was distributed in both the nucleus and the cytoplasm in small punctuate structures like the native gag–PML–RARα protein. After 48 h of treatment with RA 10^{-6} M, the original multigranular staining was replaced by a more homogeneous diffuse nuclear labelling, together with a perinuclear accumulation of the protein. In contrast, the gag–PML–RARαMW and the gag–PML–RARαMM protein localizations were mainly nuclear and finely dispersed in all the transfected cells, indicating that the mutation of cysteine 304 abrogates the speckled distribution of the hybrid product in Cos-7 cells (Figure 4A). This localization was not modified by treatment of the cells with RA.

Then we verified whether the mutations might alter the transcriptional activity of gag–PML–RARα on βRARE. The data presented in Figure 4B show that all the mutated forms were able to activate βRARE in a ligand-dependent manner, similar to PML–RARα and gag–PML–RARα. The single mutation 424 increased the transactivating properties of mutant WM, but this effect was annihilated by the second mutation 304 in the double mutant MM. We also compared the effects of wild-type gag–PML–RARα and the mutants on AP-1 activity. As shown in Figure 4B, in control experiments RA strongly reduced the AP-1 activity in cells overexpressing truncated ΔRARα. This effect is similar to that observed previously with wild-type RARα (Desbois *et al.*, 1991; Doucas *et al.*, 1993). In contrast, cells overexpressing the gag–PML–RARα product showed a low level of AP-1 activity in the absence of RA, and this level was raised following RA treatment. A similar effect was observed with PML–RARα, as reported previously (Doucas *et al.*, 1993). The gag–PML–RARαWM mutant showed the same transcriptional properties as wild-type PML–RARα, whereas the mutants MW and MM displayed the opposite effect, with an RA-dependent repression of AP-1 activity. These data show that the mutation in cysteine residue 304 has the strongest effect on the biochemical properties of the gag–PML–RARα protein with respect to its interference with AP-1 transcriptional activity.

Characterization of the haematopoietic cells transformed by PRαN

For a cytological analysis, colonies grown in semi-solid medium were picked and seeded in liquid medium. After a few days the liquid cultures showed a homogeneous population of immature blast cells characterized by very finely dispersed chromatin (Figure 5A). These cells were negative for the erythroid markers histone H5, carbonic anhydrase and haemoglobin. From their morphology, they could not be classified in a precise haematopoietic lineage but looked like the pluripotent cells transformed by the E26 leukaemia virus (Graf *et al.*, 1992).

To determine more precisely the nature of these cells, we analysed their expression of the specific membrane antigens described by McNagny *et al.* (1992). The MEP 17 antigen is expressed at a high level on E26-transformed multipotent cells, T and B lymphocytes and thrombocytes, and at much lower level on late erythrocytic, myelomonocytic and eosinophilic cells. The MEP 21 and MEP 26 antigens are present on transformed progenitor cells able to differentiate into erythrocytes, myeloblasts, thrombocytes and eosinophilic cells, but their expression is extinguished during differentiation except for on the mature thrombocytes. Moreover, they are not expressed on lymphoid cells. Therefore these antigens are characteristic of multipotent precursors. The MYL 51/2 antigen is specific for normal and transformed myelomonocytic cells and is absent from erythroid and lymphoid cells (Graf *et al.*, 1992; McNagny *et al.*, 1992). The expression of all of these antigens was investigated by a cytofluorimetric analysis on PRαN-transformed cells compared with erythrocytic cells transformed by AEV (HD4 cell line) and with either myeloid or multipotent erythroid–myeloid cells transformed by E26 (HD57M and HD57E cell lines respectively). The data are presented in Figure 5B. As expected, the E26 multipotent cells HD57E were positive for only MEP 17, MEP 21 and MEP 26 antigens. The myeloid E26-transformed cells HD57M exhibited only MEP 17 and MYL 51/2 antigens. The erythroid AEV-transformed cells HD4 were negative for all the antigens tested. The PRαN-transformed cells exhibited neither the MEP 21 nor the MYL 51/2 antigens, whereas they expressed the MEP 17 antigen at an average concentration and the MEP 26

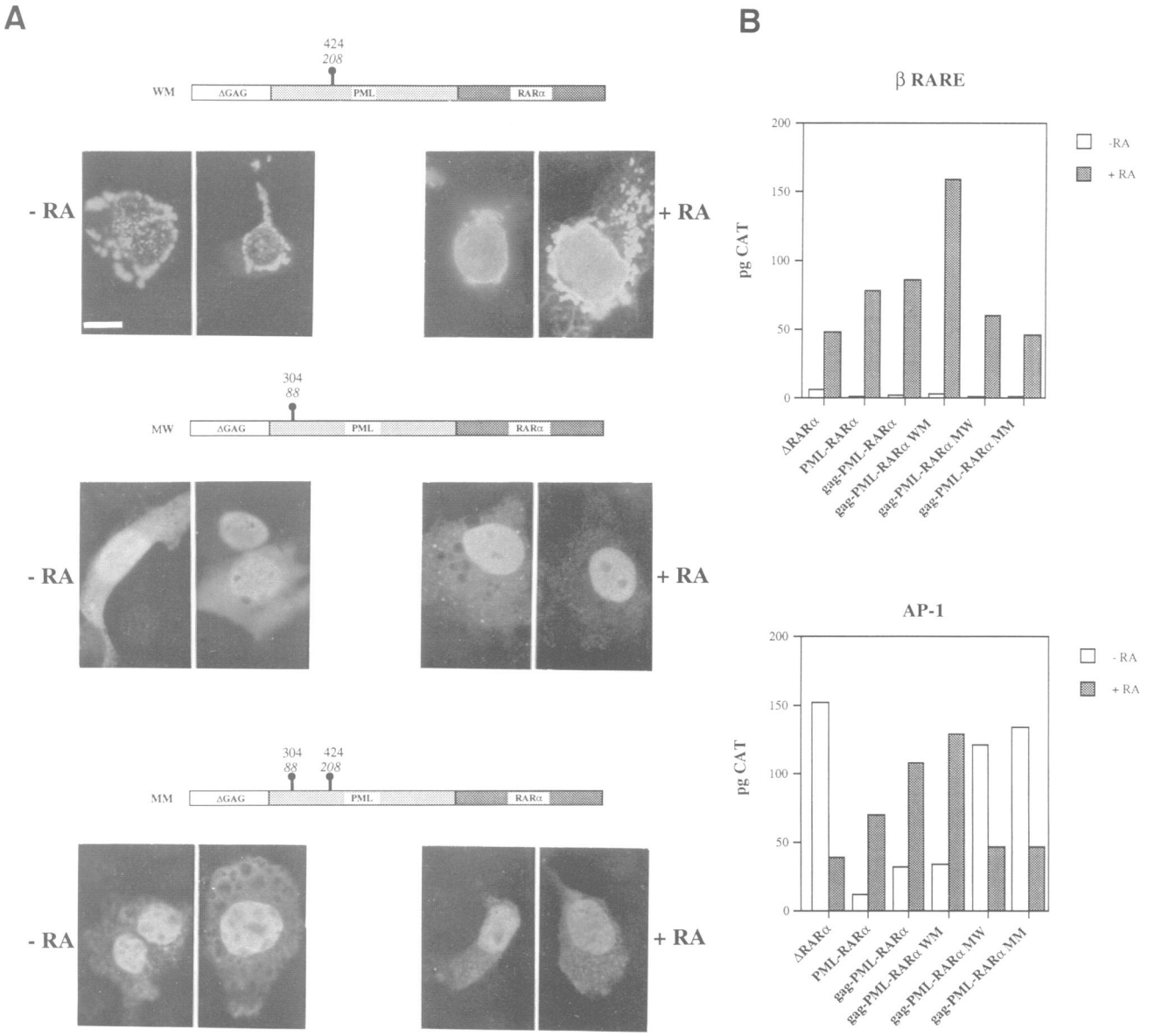


Fig. 4. Intracellular localizations and transcriptional activities of the mutated forms of gag–PML–RARα. (**A**) Localization of gag–PML–RARα WM, gag–PML–RARα MW and gag–PML–RARα MM products in Cos-7 cells. The schematic structure of each mutated form is presented above the plate. Numbering of the mutation is as in Figure 3. The proteins in Cos-7 cells were detected with an anti-PML antibody. Bar scale, 5 μm. (**B**) Transactivating properties of gag–PML–RARα WM, gag–PML–RARα MW and gag–PML–RARα MM products in HeLa cells. The direct transactivating activity on a βRARE and interference with the AP-1 activity are presented. In both cases, data are from one representative experiment out of four which gave similar data.

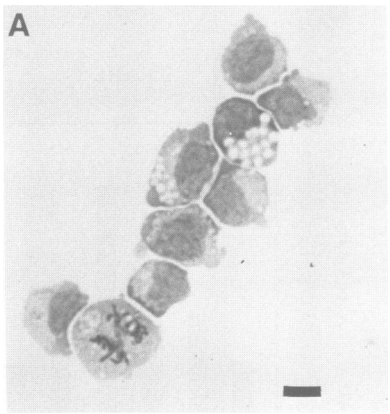
antigen at the same concentration as the multipotent E26 cells.

Treatment of the PRαN-transformed cells with RA failed to induce their differentiation. This correlated with the absence of any reorganization in gag–PML–RARα intranuclear distribution in these cells (data not shown).

When combined, these data suggest that the BMCs transformed by PRαN are very immature haematopoietic progenitors with a phenotype different from that of the cells transformed by AEV and very close to that of the E26 multipotent cells.

The PRαN retrovirus induces an acute leukaemia in vivo

To test the leukaemogenic potentials of gag–PML–RARα *in vivo*, the PRαN virus was injected intravenously into 10 day old chicken embryos. As controls, embryos were injected with the PN, PΔN or ΔRαN viruses. As shown in Table I, most of the embryos infected with PRαN developed a leukaemia within 2 weeks of hatching. In contrast, no sign of leukaemia was seen in animals infected with PN, PΔN or ΔRαN within 6 weeks of hatching. Leukaemic cells in the blood of the PRαN-infected chickens looked very similar to those developed following infection of the bone marrow *in vitro* (Figure 6A). BMCs of leukaemic chickens expressed the expected gag–PML–RARα protein, as revealed in the Western blot shown in Figure 6B. They could be grown in culture as in cells obtained from the *in vitro* infection of bone marrow. Dissection of the animals revealed no other affected organs. Therefore the gag–PML–RARα gene induces an acute leukaemia *in vivo*.



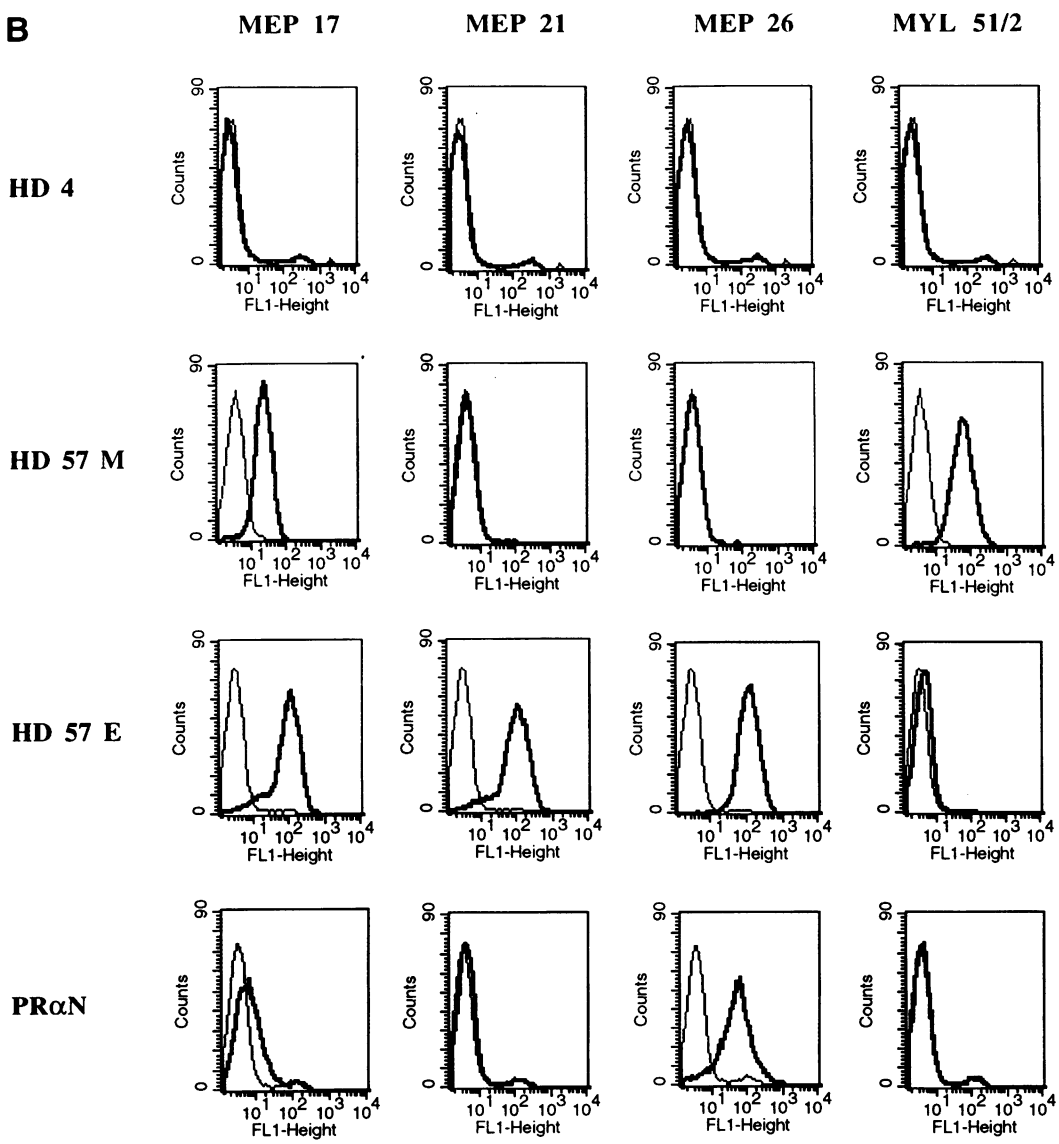


Fig. 5. Phenotypic characterization of PRαN BMCs. (**A**) Light microscopy of BMCs infected with PRαN and stained with Wright–Giemsa. Bar scale, 5 μm. (**B**) The phenotypic characterization of PRαN BMC by a FACS analysis. The expression of the cell surface antigens MEP 17, MEP 21, MEP 26 and MYL 51/2 was analysed by FACS (thick line). Non-specific fluorescence with PBS is displayed as a thin line in each panel. Details of the cells used are indicated to the left of the figure.

Discussion

Construction of an efficient retrovirus vector of PML–RARα

The purpose of this work was to investigate the oncogenic properties of the PML–RARα product. We chose an avian model of haematopoietic transformation for several reasons. First, the avian system has been widely used in the past to identify oncogenes involved in leukaemic transformation (Graf *et al.*, 1978). Secondly, previous work by some of us (C.Lavau and A.Dejean, unpublished data) and by others (Grignani *et al.*, 1993) failed to produce efficient murine retrovectors of PML–RARα that could be used to infect mammalian primary haematopoietic

Table I. Leukaemogenicity of different viruses after *in ovum* inoculation

Virus	Leukaemia incidence	Time of leukaemia detection (days)
PRαN	10/12	0, 1, 1, 1, 1, 2, 4, 7, 9, 9
PN	0/16	–
PΔN	0/16	–
ΔRαN	0/26	–

Virus suspensions were injected into the chorioallantoic veins of 10 day old chicken embryos. After hatching, the appearance of leukaemia was monitored on blood smears. The day post-hatching at which the leukaemia was detected for each leukaemic chicken is indicated.

cells. Preliminary attempts to construct self-replicating vectors carrying the active PML–RARα product were unsuccessful because the retrovectors rearranged and deleted the foreign sequence. To circumvent this instability problem, we decided to construct defective retrovirus vectors in which the PML–RARα sequence is fused to a residual gag sequence. The rationale was that most natural avian leukaemia retroviruses carry viral oncogenes in such a configuration, and that the fusion of gag residues at the N-terminus of the oncoprotein does not modify the intracellular distribution of the oncoprotein (Bishop and Varmus, 1985). Previously, we have successfully used this strategy to demonstrate the oncogenicity of a human rearranged RARβ isolated from a hepatitis B virus-induced hepatocarcinoma (Garcia *et al.*, 1993). Here we show that fusion of the PML–RARα product to the residual gag peptide does not alter its intracellular localization, its transactivating properties and its interference with AP-1. This strategy allowed us to rescue retroviruses at fairly high titres that could transmit and express PML–RARα or derived sequences.

A new model for assaying the oncogenic properties of PML–RARα in vivo and in vitro

So far, there has been no demonstration that PML–RARα is sufficient to transform haematopoietic progenitors into leukaemic cells. The effects of PML–RARα were assessed by expressing an exogenous protein in human leukaemic cell lines, whose differentiation could be tested *in vitro* (Grignani *et al.*, 1993; Rousselot *et al.*, 1994). Whereas these data showed that PML–RARα could block the differentiation, they did not demonstrate that the protein was responsible for the leukaemic transformation. Our present data on primary bone marrow progenitors *in vitro* and *in vivo* clearly demonstrate that PML–RARα, at least in its form with mutations 304 and 424, is sufficient to induce the oncogenic transformation of normal haematopoietic progenitors. PML–RARα blocks the differentiation of these cells and promotes their growth even at low concentrations of growth factor. Moreover, these cells displayed no apoptosis or growth inhibition after treatment with RA. Thus, it is likely that PML–RARα interferes with those molecular events which control cell commitment to differentiation and apoptosis in haematopoietic progenitors.

Cells transformed by PML–RARα in the chicken clearly differ from the human APL cells, produced by the t(15; 17) translocation, which are blocked at the promyelocytic

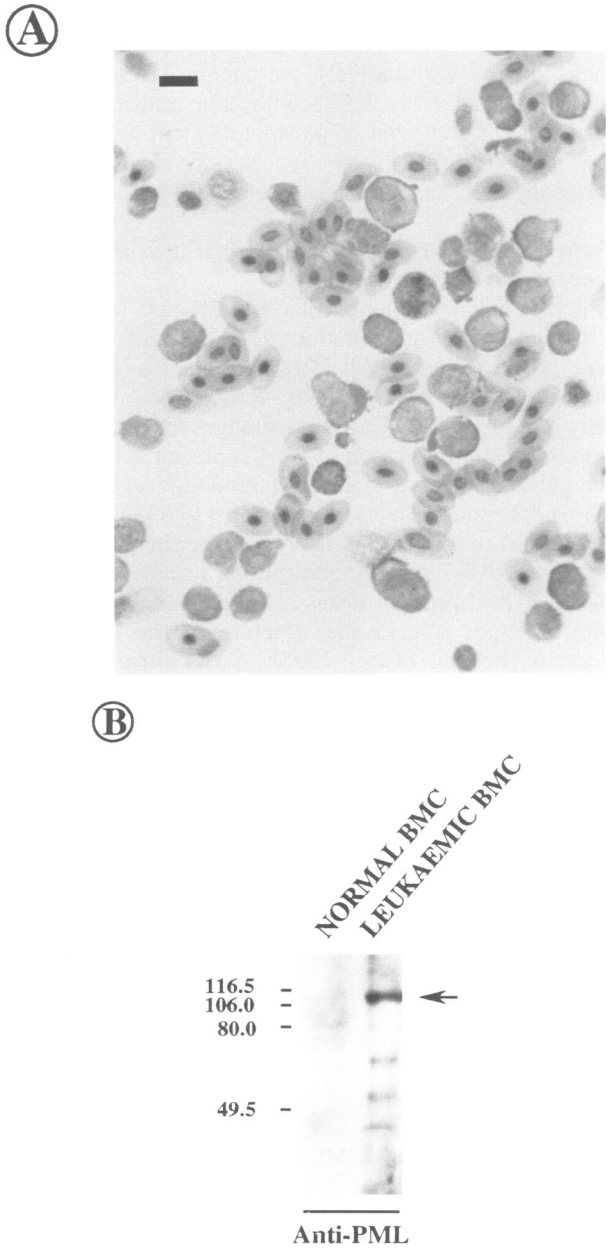


Fig. 6. Leukaemic cells in chickens infected with PRαN. (**A**) Light microscopy of a Wright–Giemsa-stained blood smear of a leukaemic chicken. Bar scale, 10 μm. (**B**) The detection of gag–PML–RARα product in BMCs from chickens infected with PRαN. 10 μg of total protein from cell extract were loaded. The Western blot was revealed by an anti-PML antibody. BMCs from non-infected chickens were used as controls.

stage. In the chicken, the cells exhibit cytological features and membrane antigen patterns of very immature cells similar to the pluripotent haematopoietic progenitors obtained after transformation by the E26 virus (Graf *et al.*, 1992). Consequently, these cells are different from the erythrocytic progenitors blocked by v-erbA and from the myeloid progenitors blocked by v-myb.

The reason why the chicken cells transformed by PML–RARα are different from the human APL cells is unknown. The specific phenotype of the chicken cells might be a result of the two point mutations present in gag–PML–RARα. Another possible explanation is that in human

APL, the t(15;17) translocation takes place after the pluripotent stem cells have been committed to the myelocytic lineage. Alternatively, we cannot exclude the facts that in human APL the chromosome translocation may take place at the level of pluripotent stem cells, and that the block of differentiation would only become effective when the cells reach the promyelocyte stage.

Role of the mutations in PML–RARα in the oncogenic transformation

Chicken haematopoietic cells transformed by the virus PRαN carry the two mutations at amino acid residues 304 and 424 in the gag–PML–RARα sequence (positions 88 and 208 respectively in PML–RARα). We suggest that they provide some selective advantages for the transformation or the multiplication of BMCs, presumably in making the cells insensitive to the differentiating effects of RA. The biochemical effects of mutation 424 remain unknown. In contrast, mutation 304 is able to alter both the interference with AP-1 activity and the intracellular localization of the hybrid protein; it has also been shown recently to play a major role in the structure of the PML RING domain (Borden *et al.*, 1995). A definitive conclusion concerning the role of these two mutations in altering gene expression in transformed cells awaits the identification of the correct target genes in these cells.

Mutations at positions 88 and 208 have not been found in the PML–RARα cDNAs cloned to date from human APL cells. One may speculate that in APL another genetic event takes place which alters the molecular pathways more or less directly connected to the mutated domain of PML. Interestingly, the mutation at position 424 (208 in PML–RARα) removes a serine, which might be a putative phosphorylation site. One may imagine that the phosphorylation of this site is abrogated in APL cells. Hence, our data could suggest that additional events cooperate with the formation of the PML–RARα hybrid product to generate APL in humans.

In contrast to human APL cells, the chicken leukaemic cells transformed by PML–RARα are resistant to the differentiating effect of RA. In human APL blasts, the PML nuclear bodies are disrupted because of the presence of the PML–RARα hybrid. It has been suggested that the therapeutic effect of RA could be related to its ability to relocalize the PML–RARα hybrid and to restore intact PML nuclear bodies (Dyck *et al.*, 1994; Koken *et al.*, 1994; Weis *et al.*, 1994). In chicken BMCs transformed by gag–PML–RARα, no such redistribution of the PML–RARα product was visible following RA treatment. This observation may be correlated to the inability of the chicken transformed cells to differentiate following exposure to RA. This lack of response is likely to result from point mutation 304 in the fusion protein because the gag–PML–RARαMW mutant does not show any change in its intracellular distribution following RA treatment when tested in Cos-7 cells. However, we cannot exclude an additional role for mutation 424 in this phenomenon in bone marrow transformed cells.

Several cases of relapse resulting from cell resistance to the therapeutic effect of RA have been reported in APL patients (reviewed in Warrell, 1993). In the light of our data, it will prove interesting to check for point mutations in the PML–RARα product in RA-resistant APL patients. The chicken model we have developed here may then prove helpful in elucidating these mechanisms of resistance and finding new therapeutic approaches.

In conclusion, our work has provided the first demonstration that PML–RARα, at least in its form with mutations 304 and 424, is directly responsible for the oncogenic transformation of haematopoietic progenitors. Our work opens the way to investigating the molecular mechanisms underlying oncogenic transformation by this rearranged receptor.

Materials and methods

Viruses

All virus genomes were constructed from an AEV-based retroviral vector containing a Neo^R gene, as described previously (Garcia *et al.*, 1993). The long forms of PML–RARα (de Thé *et al.*, 1990; PML–RARα L), the PML-L (Kastner *et al.*, 1992), PML-L-Δ and ΔRARα sequences, were fused in-frame with the gag retroviral sequence, as shown previously (Garcia *et al.*, 1993). For convenience, appropriate cloning sites were generated in the gag sequence. All these constructs on fusion with gag retained the first 647 nucleotides of the gag coding sequence. Details of the construction can be provided on request. For virus production, the respective plasmids carrying the virus genomes were transfected on CEFs with a plasmid carrying the genome of the helper virus RAV-1. Rescued viruses were collected from the supernatants after several passages of the cultures, and then titrated on CEFs by the induction of resistance to G418 (Garcia *et al.*, 1993). The titres of viruses PRαN, PΔN, PN, ΔRαN and TXN were respectively 10^5, 2×10^5, 9×10^4, 2×10^5 and 4×10^5 r.f.u./ml (resistance forming units/ml).

Infection of BMCs

Fresh BMCs from 17–19 day old chicken embryos were infected by cocultivation for 24 h with infected and G418-selected CEFs. They were then seeded in methyl cellulose culture containing G418 at 3 mg/ml, as described previously (Gandrillon *et al.*, 1989). After 7 days, colonies were picked and seeded in liquid culture containing αMEM supplemented with 20% fetal bovine serum, 5% chicken serum and 5% bovine serum albumin (Sigma). Under low-serum conditions, the culture medium contained αMEM supplemented with 5% fetal bovine serum, 1% chicken serum and 2.5% bovine serum albumin.

Transfection of cells and CAT assays

The CEFs were stably transfected by using the polybrene method described previously (Gandrillon *et al.*, 1989). HeLa cells and the Cos-7 cells were transfected using a calcium phosphate procedure, as described in Desbois *et al.* (1991). Assays for AP-1 activity and the activation of βRARE–CAT reporters were as described previously (Desbois *et al.*, 1991). The effects of either gag–PML–RARα or PML–RARα were tested by transfection of the respective expression plasmids. The CAT enzymatic activity was measured by using a CAT-ELISA kit (Boehringer-Mannheim, Germany).

Flow cytometry analysis

An analysis of the membrane antigens was performed on freshly transformed BMCs. The MEP 17, MEP 21, MEP 26 and MYL 51/2 cell surface antigens (McNagny *et al.*, 1992) were detected after incubation for 30 min at room temperature with the respective antibodies (kindly provided by T.Graf, Heidelberg, Germany), diluted 1:200. Fluorescein isothiocyanate-labelled goat antibody (BioSys, France), diluted 1:100, was used as the second antibody. A negative control experiment was performed with the second antibody alone. Antigen-positive cells were counted on a 10 000 cell sample using a Becton-Dickinson FACStar Plus flow cytometer at 488 nm with a DF 530/30 filter (Ion Laser Technologies). Data acquisitions and analyses were performed using Lysis II software on a consort 340 computer (Hewlett Packard).

Protein analysis

Immunofluorescence. Transfected Cos-7 cells or infected chicken cells were grown in microwell plates for adherent cells or were cytospun on glass slides for non-adherent cells. The immunostaining was performed as described by Kastner *et al.* (1992). In some cases, the cells were stained further with Hoechst 33258 at 10 μg/ml. The cells were

examined and photographed using an Olympus Vanox AH-2 fluorescence microscope.

Western blotting. Cells were harvested from cultures, washed in PBS and lysed in 1% final SDS lysis buffer. For the immunodetection of the PML–RARα protein, cells lysates were concentrated as described by Rochette-Egly *et al.* (1991). After electrophoresis, the samples were transferred to a nitrocellulose membrane and enhanced chemiluminescence Western blotting was performed (Amersham, UK).

PCR and genomic sequencing

Amplification of the provirus DNA sequences was performed using 500 ng *Bam*HI-digested cell genomic DNA. After heat denaturation for 5 min at 97°C, 1.25 U of AmpliTaq polymerase (Perkin-Elmer, Norwalk, CT) were added and the PCR was run for 40 cycles. Each cycle was performed for 30 s at 95°C, 20 s at 65°C and 1.5 min at 72°C. Between one and three identical PCR amplifications run in parallel were pooled, purified using the Wizard™ PCR Preps DNA purification system (Promega, Madison, WI) and sequenced with 3.2 pmol primer using the PRISM™ Ready Reaction kit and the 373A DNA sequencer (Applied Biosystems Inc., Foster City, CA).

In vivo leukaemogenicity assay

Some 100–200 μl of freshly harvested virus were injected into the chorioallantoic vein of 10 day old embryos. After hatching, the animals were monitored twice a week for leukaemia by preparing blood smears that were stained with Wright–Giemsa.

Cell lines and antibodies

The HD57E, HD57M and HD4 cell lines, and the anti-MEP 17, anti-MEP 21, anti-MEP 26 and anti-MYL 51/2 antibodies, were kindly provided by T.Graf (Heidelberg, Germany). The anti-RARα antibody was kindly provided by P.Chambon (Strasbourg, France). The anti-PML antibody has been described previously (Weis *et al.*, 1994).

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